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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

BIOVATION LIMITED
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ABERDEEN SCIENCE PARK
BALGOWNIE DRIVE
ABERDEEN AB22 8GU

Patents ADP number *(if you know it)* 7527534001

If the applicant is a corporate body, give the country/state of its incorporation (INCORP. IN SCOTLAND)

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4. Title of the invention

PROTEIN ANALYSIS

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5. Name of your agent *(if you have one)* —

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

KILBURN & STRODE
20 RED LION STREET
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PROTEIN ANALYSIS

The present invention relates to methods for analysing mixtures of proteins. In particular, the invention relates to methods to compare proteins between different cells and tissues. The invention involves the combination of protein digestion or cleavage, and subsequent analysis of mass. The invention also optionally involves the fractionation of proteins or peptide fragments.

Current method to analyse *en masse* complex mixtures of proteins such as in mammalian cells or tissues require that the proteins are separated by technologies such as two dimensional (2D) gel electrophoresis. For this technology, cellular proteins are usually separated on the basis of charge in one dimension and on the basis of size in the other dimension. Proteins can either be identified with reference to the electophoresis migration pattern of a known protein or by elution of the protein from the electrophoretically separated spot and analysis by methods such as mass spectrometry and nuclear magnetic resonance. However, limitations of the 2D protein gel method include the limited resolution and detection of proteins from a cell (typically only 5000 cellular proteins are clearly detected), the limitation to identification of separated proteins (for example, mass spectrometry usually requires 100fmoles or more of protein for identification), the specialist nature of the technique and the difficulty in automating the technique in order to achieve very high protein analysis throughputs. There is thus a need for superior methods to analyse complex mixtures of proteins *en masse* especially using methods without gel electrophoresis and methods which are easy to automate.

The core of the present invention is that proteins are either digested or cleaved into smaller peptide fragments and then analysed by mass spectroscopy. Optionally, there will also be one or more protein or peptide fractionation steps to limit the complexity of the protein or peptide mixture being subject to measurement of mass analysis typically as mass-to-charge ratio measured by mass spectroscopy. Optionally, proteins or peptide fragments may also be conjugated with a "chemical tag" to assist in fractionation.

In one embodiment, the invention provides for cleavage of proteins using proteases or chemical methods and subsequent mass analysis. In this case, the analysis of protein mixtures is assisted by sequential cleavage cycles whereby the spectrum of proteins and peptides are analysed following each cleavage cycle. This method could also include chemical tagging cycles between cleavage cycles to increase the mass or steps to remove side-groups such as carbohydrate groups in order to reduce mass. If the mass of the range of protein fragments is then determined at the end of each cycle (either chemical tagging, cleavage or other modification), then a range of mass distributions will be obtained for each cycle. With an appropriate range of tagging, cleavage or other mass modification cycles, the result for a single protein or a mixture will be a spectrum of protein/peptide fragments some of which are altered at specific cycles. The appearance and disappearance of a particular protein/peptide fragment of a certain mass following a specific chemical tagging, cleavage or other modification cycle will provide information of certain properties of that fragment and may aid in determination of the fragment sequence. Comparison of

the spectrum of protein/peptide fragments from different related samples then allows for the identification of protein/peptide fragment differences between these samples. Particularly useful in this embodiment of the present invention is proteases which specifically recognise two amino acids and cleave the protein as a result. An example of such proteases are the prohormone convertases which cleave between dibasic amino acid pairs.

In another embodiment of the present invention, proteins or peptides are fractionated prior to mass analysis. This is conveniently achieved by using affinity reagents such as monoclonal antibodies to isolate specific proteins for subsequent mass analysis. For such analysis of larger mixtures of proteins, panels of mixed monoclonal antibodies such as those provided by recombinant libraries of antibody variable region fragments (including single-chain antibodies) are preferred in order to isolate subsets of proteins for subsequent analysis. Such panels of monoclonal antibodies will include a wide range of protein specificities which could be achieved, for example, by pre-absorbing antibody libraries on the mixed protein sample of interest and then using individual or mixtures of the selected antibodies in order to isolate subsets of proteins. Such analysis provides mass spectra for a range of different protein fractions thus facilitating detection of differences in specific proteins between samples. .

As an alternative to the use of affinity reagents which bind to specific sequences or structures in the proteins within a mixture, the invention also provides for the chemical tagging of proteins or peptides whereby such chemical tags are themselves recognised by affinity reagents which are then used to isolate the tagged proteins or peptides. In particular, the invention also provides for chemical tagging of either or both of the N or C termini such that only N or C terminal peptides are subsequently isolated using the affinity reagents. The invention also allows for sequential conjugation of different chemical tags to the protein / peptide mixture especially where N or C termini are sequentially exposed by specific cleavage of the protein / peptide and whereby the N or C termini (or both) are conjugated with a specific chemical tag upon exposure of that termini. This aspect of the invention therefore provides for a series of protein fractions with a range of conjugated chemical tags introduced at the termini, such fractions being isolated using an affinity reagent which binds to the tag. As an alternative to a chemical tag at the terminus of the protein molecule, chemical tags can also specifically be attached to non-terminus amino acids such that internal peptides can be isolated via an internal chemical tag.

Therefore, the invention provides for novel ways of analysing protein mixtures using a combination of protein digestion or cleavage and mass analysis. For large protein mixtures, particularly those isolated directly from whole cells or tissues, the additional use of affinity reagents to fractionate proteins or peptides is required in order to reduce the complexity of mixtures subjected to mass analysis. Whilst affinity reagents can be used which recognise sequences or structures in the proteins/peptides directly, this will itself require a complex library of affinity reagents such as an antibody library and therefore the additional use of chemical tags to provide moieties recognised by a set of affinity reagents provides a simpler means of using such reagents.

A further advantage of the use of chemical tags is that the subsequent fractionation of peptides by affinity reagents can greatly reduce the number of selected peptides from a protein molecule with the rest of the molecule thus being eliminated from the mass analysis. An especially convenient method for such selective chemical tagging is to initial tag either (or both of) the N and C terminus of the protein molecules in the mixture and then to digest or cleave the protein molecules with a reasonably selective reagent such as a amino acid or sequence-specific protease (such as endopeptidase Arg-C) or cleavage reagent (such as acid pH to cleave at Asp-Pro). Using an affinity reagent, N or C terminal peptides (or both) from the original protein could then be isolated and all internal peptides discarded. This reduction in complexity is then sufficient for mass analysis especially using HPLC coupled to a tandem mass spectrometer to analyse the peptides *en masse* in order to identify the individual peptides from the mixture. Alternatively, chemical tagging could be performed only after digestion/cleavage, for example with the dibasic cutters, the prohormone convertases. This would provide for tagging only at one or more internal sites of the original proteins. If the protein mixture is then subjected to a second digestion/cleavage step with a different enzyme or cleaving reagent, then the size of the tagged peptides would be reduced where a cleavage site was present in the original protein. The tagged peptides could then be fractionated using an affinity reagent and subjected to mass analysis.

In another embodiment of the current invention, a protein mixture is subjected to cycles of tagging, digestion/cleavage and mass analysis, whereby mass analysis is performed only on an aliquot of the mixture resultant from use of an affinity reagent binding to the specific chemical tag and whereby the master mixture is then subjected to tagging with a different chemical tag and digestion/cleavage. This provides sequentially a range of different fragments for mass analysis. Another variation on the method involves the same initial steps as above but, having exposed new N and C termini after cleavage, one (or both) of these new termini can then optionally be tagged with a different chemical which thus tags internal sites in the original protein. If required, the process could be repeated one or more times with a different protease or cleavage reagent, each time with the addition to the N or C terminus of a different chemical tag. In one format of the method, the whole mixture of proteins would first be tagged with two different chemical groups at each of the N and C terminus and then cleaved with a protease, such as one which specifically cuts adjacent to a specific amino acid, and tagged again at the new N and C termini with two further different chemical groups. This would result in a mixture of peptides each with chemical tags at the termini. As the N and C terminal peptides would have a specific tag, these could then be isolated from the mixture using appropriate affinity reagents. Internal peptides without either the initial N or C terminal tags could be isolated using their specific tags. The process of digestion and tagging could then be repeated to create further peptides with tags. Using specific combinations of affinity reagents for specific tags, N or C terminal or specific internal peptides from the original protein could then be isolated and selected peptides discarded to achieve a reduction in complexity sufficient for mass analysis.

Mass analysis of proteins and peptides by the present invention is preferably performed using mass spectroscopy. In particular, recent methods of interfacing liquid chromatography separation methods (such as HPLC) with tandem mass spectroscopy has already permitted the mass spectrum analysis of protein mixtures comprising up to 200 proteins. As such proteins are analysed following protease digestion, if an average ten peptides per protein is assumed, then the method can analyse up to 2000 peptides. Using methods of the present invention whereby, for example, only tagged N terminal peptides are analysed, then up to 2000 N terminal peptides derived from up to 2000 proteins could be analysed at any one time. As this is not sensitive enough for an *en masse* analysis of mammalian proteins from cells (typically 50,000 per cell), then peptides have to be segregated into at least 25 fractions in order for these fractions all to be analysed. Such further fractionation can be achieved by the direct use of affinity reagents to label internal ends after successive protein digestion/cleavage steps following which specific affinity reagents are used to fractionate peptides according to their tags. As an alternative to standard mass spectroscopy, MALDI (matrix-assisted laser desorption/ionisation) can be used to produce protein mass profiles which can be compared for protein mixtures from different cells.

Chemical tags are typically moieties which can be covalently attached to proteins usually at the N or C terminus. For chemical tagging of the N terminus, this is commonly undertaken at the terminal amine group. If it is necessary to avoid tagging of the ϵ-amino group of lysines, then these can be initially blocked using reagents such as citraconic anhydride or methyl acetimidate. Terminal amine groups are then reactive with a wide range of chemical reagents especially using succinimide esters. Such esters of common antibody-recognised ligands such as dinitrophenol and fluorescein can then attach these to the N terminus for subsequent fractionation using an antibody affinity reagent. For chemical tagging of the C terminus, methods based on carbodiimide activation are commonly used to introduce ligands which are bound by affinity reagents. Alternatively, addition of moieties to the C terminus of proteins has been described using reverse proteolysis whereby certain proteases can work in reverse to add chemical tags. It will be recognised that a wide range of internal amino acids can also be chemically tagged including Lys via the ϵ-amino group, Glu / Asp via the carboxyl group, Cys via the thiol group, Ser / Thr via the hydroxyl group and Tyr via the hydroxyphenyl group. Specific derivatisations of most other amino acids have been described. It will also be recognised that post-translation protein modifications can be used for addition of chemical tags especially with glycosylation where the sugar residues are commonly oxidised by periodate to form aldehyde groups which can then react with amine-containing molecules. Other modifications which can be used to add chemical tags include lipidation, phosphorylation and metal ion addition. It will be recognised that there are a large number of methods in the art for introducing one or more chemical tags at specific sites within protein molecules or peptides.

Affinity reagents for use in the present invention are commonly monoclonal antibodies. For specific sequences or structures within proteins or peptides, a library of recombinant antibody binding sites usually in the form of Fab's, Fvs or single-chain Fv's is used where

commonly the antibody binding sites are "displayed" using, for example, bacteriophage or ribosome complexes such that the gene encoding individual antibody binding sites can be recovered. For use in the present invention, libraries of antibody binding sites can be dispersed into groups, for example by picking and arraying phage plaques or picking and arraying genes in vectors for ribosome display. Such pools will usually contain antibody binding sites for several proteins or peptides such that the pools can be used for fractionation. Alternatively, the protein or peptide mixture to which libraries of antibody affinity reagents are required can be immobilised and used as the target for the pre-selection of suitable affinity reagents which are then dispersed into pools or used as individual reagents. For chemical tags, individual monoclonal antibodies are used to specifically bind to individual tags in order to achieve subsequent fractionation.

The various embodiments of the invention cover combinations of protein digestion/cleavage and mass analysis with optional steps of fractionation using affinity tags for specific sequences or structures in the proteins or peptides, and chemical tagging with fractionation by virtue of these tags. The different embodiments encompass different sequences of these steps as follows;

embodiment 1 - repeated digestion/cleavage cycles and mass analysis
embodiment 2 - digestion/cleavage, fractionation with affinity reagents, mass analysis
embodiment 3 - fractionation with affinity reagents, digestion/cleavage, mass analysis
embodiment 4 - terminal chemical tagging, digestion/cleavage, fractionation with tag affinity reagents, mass analysis
embodiment 5 - as 3 but with additional cycle(s) of tagging, digestion/cleavage, fractionation
embodiment 6 - repeated tagging, digestion/cleavage cycles and mass analysis

The current invention should be considered to encompass these and related protein/peptide processing steps with the core objective of reducing the complexity of protein mixtures in order to achieve mass analysis of the resultant protein/peptide fractions.

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